# THE CULTURE OF *BABESIA (PIROPLASMA) CANIS* IN VITRO

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Plate XLII

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## INTRODUCTION

The present paper contains the preliminary results of the authors' investigations on the cultural forms of *Babesia canis*. The importance of the investigation of cultural forms of Protozoa does not need emphasis, and many practical applications are suggested, but in this paper we confine ourselves to some remarks on the morphology and life-history of the Sporozoön as seen in the culture tube. The method of C. C. Bass (1912) has been followed, without any addition or modification, though the parasite is not so easily cultivated as the Plasmodia of man.

## TECHNIQUE

Cultivation succeeded in two out of four attempts. In each successful case the infected animal, whose blood was used, was a puppy about three months old. The blood used in the first culture was taken from the heart on the fifth day after inoculation, in the second case on the fourth day after inoculation.

Ten c.c. of heart blood, drawn with aseptic precautions, was mixed with 1/10 c.c. of a 50 per cent. aqueous solution of Merck's glucose. The blood was carefully and gently defibrinated by means of a rod, and the clot was removed. It was noticed that the amount of clot removed was much in excess of that taken from a similar quantity of human malarial blood. The defibrinated blood was distributed into smaller tubes, placing about one inch of liquid in each tube. No centrifugalisation was necessary. The tubes were incubated at 37° C. The corpuscles settled to the bottom in a short time, leaving a layer of serum above.

Marked haemolysis was seen in all the cultures attempted. It was found advantageous to take the blood of the puppy before the crisis, that is, before too many parasites were present in the peripheral or heart blood.

No sodium citrate nor ascitic fluid was added to the cultures, as was done by Ziemann (1913), nor citrate and saline as used by Toyoda (1913).

## PROGRESS OF THE CULTURES

We will set forth in some detail the progress of events in our FIRST CULTURE, which was more successful than the second, in that more divisions occurred in it.

In the original heart blood, before inoculation, the parasites were not very numerous, pairs or singles being found in the infected red blood corpuscle (Pl. XLII, fig. 1). Only one group of four parasites was seen in any corpuscle.* The Babesia were chiefly pyriform in shape, with small compact nuclei, and the secondary

* Graham-Smith (1905) found that the infected red blood corpuscles containing more than four *Babesia canis* constituted less than 0·3 % of the total. He counted 22,589 infected corpuscles from peripheral and heart blood of dogs. Corpuscles containing one and two parasites formed 96·4 % of the total. (*Journ. Hygiene*, V, p. 252)

loose mass of chromatin was not well marked. Phagocytosis was evident, one large mononuclear leucocyte had ingested four infected corpuscles (Pl. XLII, fig. 2).

After 7 *hours* large numbers of intracorpuscular parasites occurred in clumps, especially at the margins of the smears. The infected corpuscles usually contained four parasites each (Pl. XLII, fig. 3), a few contained eight merozoites (Pl. XLII, fig. 4). Hence the original parasites had divided once. The piroplasms, usually pyriform, now showed clearly the secondary loose mass of chromatin. Examples of division by budding and chromatin forking (Pl. XLII, fig. 5), as described by Nuttall and Graham-Smith (1907) from dog's blood, were seen.

After 15 *hours* numerous infected corpuscles contained four parasites, some corpuscles contained eight, while a very few showed sixteen merozoites (Pl. XLII, fig. 7).

At 24 *hours* there was an increase in the number of infected corpuscles containing eight parasites, as well as in those containing sixteen (Pl. XLII, fig. 8). Corpuscles containing four piroplasms were also present. Rounded dividing forms, exhibiting chromatinic forking were found. There was thus evidence of another division beginning.

After 30 *hours* some parasites were seen to be degenerating. Corpuscles containing eight living piroplasms were numerous. The parasites seemed to have grown larger.

After 48 *hours* numerous corpuscles were found to contain sixteen merozoites (Pl. XLII, fig. 9). One cluster of 29 was found. Three of the parasites in this group each showed two chromatinic dots, and were probably about to divide, so that the cluster represented 32 daughter forms derived from one parent Babesia. The host corpuscle had burst, but its remains could be distinguished.

At 60 *hours* the parasites were few and were smaller in size. Two groups of eight and one of four were noticed in one smear. These parasites had circular chromatin masses. No others were seen on this smear. Most of the cultural piroplasms were now dead, and at 68 hours none was found.

Three divisions had occurred in the cultures, from twos, through fours to eights and sixteens in infected red blood corpuscles.

Heart blood of the dog, kept as control, and incubated at 37° C.,

showed a few somewhat shrunken parasites after 24 hours, and no parasites after.

In our SECOND CULTURE only pairs or single pyriform parasites were found in the heart blood before incubation. This culture progressed more slowly than the former.

After 6 *hours'* incubation there was more variety in the form of the parasites, some being larger and round and some amoeboid.

At *7 hours* a very few parasites were showing the commencement of gemmation.

After $8\frac{1}{2}$ *hours* some free pyriforms were found and an intracorpuscular group of four. The nuclei of these contained small dots (karyosomes) and a little loose chromatin.

At 16 *hours* fours were more numerous, and distinct chromatinic budding was seen in several specimens.

After $18\frac{1}{2}$ *hours* there were numerous groups of four pyriform parasites, a few eights and one group of twelve. The loose chromatin was well marked. There was evidence of various types of division. Some degenerating forms were now noticed.

At $23\frac{1}{2}$ *hours* corpuscles were seen containing one, two and four parasites.

At $24\frac{1}{2}$ *hours* groups of four merozoites were fairly common.

At 30 *hours* many fours were present and some groups of eight.

At 41 *hours* groups of fours were still found, and $\frac{1}{2}$ c.c. of the culture, containing most of the corpuscles, was inoculated into a young puppy. The puppy developed piroplasmosis and succumbed five days later. The remains of the culture were examined at about 66 hours and no live parasites were seen.

There is thus evidence that only two divisions occurred in this culture. It certainly did not grow so well nor so rapidly as the first. We are unable to explain the cause of this difference in the progress of the two cultures, but we may remark that the strain in the second case was of a more chronic character.

## MORPHOLOGY OF THE CULTURAL FORMS

The piroplasms were examined fresh, and after fixation and staining. Smears were made from time to time and fixed by the wet and by the dry methods, and stained by Romanowsky, Giemsa

and haematin stains. Bouin's fluid was used in some cases as a fixative.

In this paper we do not propose to deal exhaustively with the morphology of the cultural forms of *Babesia canis*, but to record only the more important findings.

The parasites exhibited marked variation in shape. Pyriform, amoeboid, round and oval types were seen (Fig. 1, A—D). The method of gemmation and chromatinic forking was observed, as first described by Nuttall and Graham-Smith from the blood of the dog. There was also evidence of binary fission.

The pyriform piroplasms (Fig. 1, A—B) usually exhibited a distinct nucleus, as a dot of chromatin, often surrounded by a clear achromatic halo. Such a nucleus is of the karyosomatic type, the chromatinic dot representing the karyosome. Such a structure has

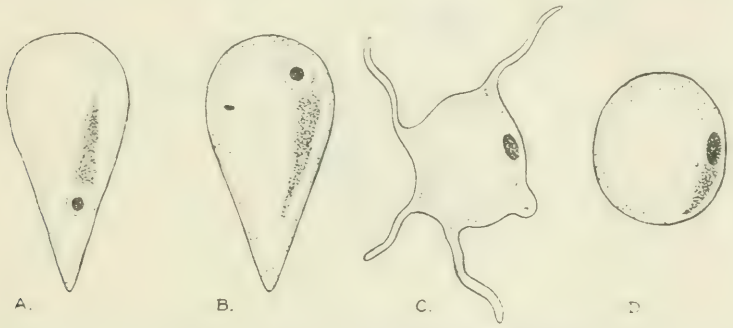


Fig. 1. Various forms of *Babesia canis* in culture.
A, B. Two pyriform parasites showing variation in nuclear position. Loose chromatin also present.
C. Amoeboid form. D. Rounded form.

been recorded in *Babesia canis* by Schuberg and Reichenow (1912). The presence of a very thin nuclear membrane is sometimes suggested, but at other times such a nuclear membrane is certainly not well marked. A secondary mass of loose chromatin, of a reticulate or 'woolly' character is also seen, as described from blood by Nuttall and Graham-Smith and by Christophers (1907) in *Babesia canis*, and by one of us (Fantham, 1907) in *Babesia bovis*. The secondary mass of chromatin was well seen in 7-hour cultures. In some pyriform parasites a very small dot of chromatin was observed (Fig. 1, B), the so-called blepharoplast of Schaudinn and Lühe;

but this punctiform chromatinic mass is not comparable with the blepharoplast of a flagellate.

Pyriform Babesia in cultures of 7 hours' duration had the nucleus usually near the pointed end (Fig. 1, A), while after 24 hours' culture it was sometimes seen to be near the rounded or blunt end of the parasite (Fig. 1, B). Thus the position of the nucleus may vary in different specimens.

Some amoeboid forms with fine pseudopodia were present, especially in 7 hour cultures (Fig. 1, C). Such parasites were also found, motile, on examining fresh preparations.

Rounded forms often showed signs of division by the method of gemmation.

FIG. 2. Infected corpuscle containing four parasites. The upper one of the four is oval, and is Leishmania-like; the lower three show masses of loose chromatin.

In one case, in a 15 hours' culture, a remarkable Leishmania-like oval form was seen, with nucleus and blepharoplast (Fig. 2, oval parasite).

## DIVISION OF PARASITES IN VITRO

Many examples were found of the mode of gemmation with chromatinic forking (Figs. 3, 4), now made a diagnostic character of the genus Babesia. Round piroplasms protrude two small buds symmetrically arranged to one side (Fig. 3, C), which buds contain chromatinic cores connected with the main nucleus (Fig. 3,

D—G). In some cases the buds are almost entirely composed of chromatin at first, and seem as if they are about to separate from the parent. The buds then grow at the expense of the rounded

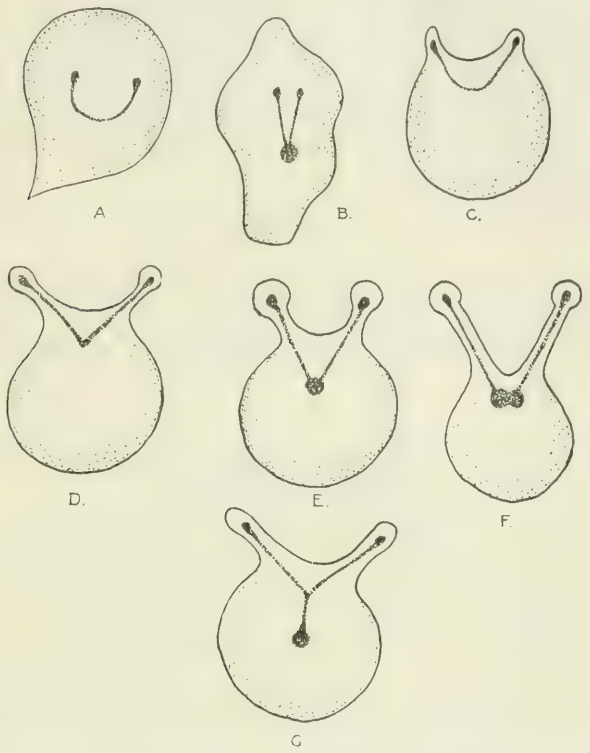


Fig. 3. Babesia showing division by gemmation, accompanied by chromatin budding and forking.

A. Parasite with arcuate chromatin.

B. Somewhat amoeboid parasite with chromatin fork.

C. Shows small, symmetrical cytoplasmic buds and chromatin processes entering buds.

D. Parasite with larger buds; main chromatin not distinguishable, but chromatin cores well marked.

E. Larger buds shown. Well marked main chromatin mass and processes.

F. Parasite showing commencement of fission of main chromatin mass.

G. Form showing typical Y shaped chromatin bifurcation.

portion of the parent parasite, and the linear processes of chromatin give rise to the loose mass of secondary chromatin seen in the daughter pyriform piroplasms. Early stages of chromatinic

budding and forking were seen while the parasite was still round or somewhat amoeboid (Fig. 3, B) in contour. Various stages of division may be observed concurrently in several parasites in one corpuscle (Fig. 4). Certain cases were noticed in which the chromatinic forking did not assume the typical Y form (Fig. 3, G), but was arcuate in character (Fig. 3, A). Sometimes it is difficult (*vide* Fig. 3, D) to observe the main chromatin mass from which the forks arise. It is unnecessary to enter into a lengthy description of this mode of division, as it has already been described by various

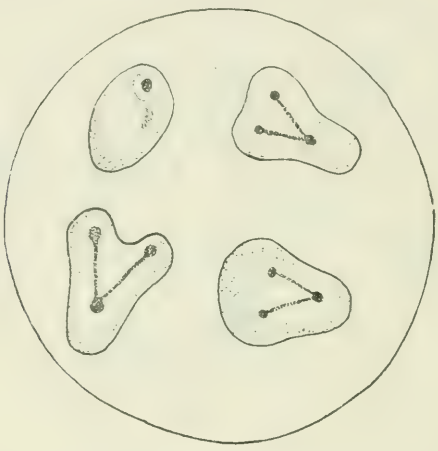

Fig. 4. Infected corpuscle showing parasites in various stages of division.

authors from the blood of dogs. The present is the first record of its occurrence in cultures, and we also succeeded in seeing the method in operation in living parasites taken from cultures.

Christophers (1907) considers that, beside the mode described above, there is also division by direct binary fission. Nuttall and Graham-Smith have recorded such direct division in the case of rounded blood forms of *Babesia canis*. We agree with the above-mentioned workers, as parasites of various shapes are often seen with two and even four principal chromatinic masses (Fig. 5, A—B). We have also seen parasites whose nucleus exhibited a form of promitosis. Further, we have observed long, somewhat sausage-shaped parasites, containing two chromatin masses, and others

indented in the middle, apparently dividing into two pyriforms, as figured by Christophers (1907, p. 21).


Fig. 5. Two parasites showing direct division. A has two nuclei, B has four chromatin masses.

The free forms of the piroplasms, seen in cultures, which were attempting to enter fresh blood corpuscles, were invariably of the pyriform type, and were endeavouring to enter by the blunt or rounded ends.

## SUCCESSFUL INOCULATION OF THE ANIMAL HOST FROM A 41 HOURS' CULTURE

A young puppy was inoculated successfully with about $\frac{1}{2}$ c.c. of a 41 hours' culture of *Babesia canis*. The inoculation was performed intraperitoneally. The puppy showed numerous parasites on the early morning of the fifth day after inoculation, and died on the evening of the same day.

Unfortunately, the removal of a series of portions of the culture for examination, has hitherto left us with insufficient material to attempt sub-cultures. We have, however, no doubt that such sub-cultures would be successful, as was shown by Ziemann (1913).

## SUMMARY

1. We have succeeded in cultivating *Babesia* (*Piroplasma*) *canis* in two out of four attempts, following the method of Bass, using blood and glucose, and incubating at 37° C.

2. In one of these cultures, starting with heart blood containing corpuscles infected with one, two or, exceptionally, four piroplasmata, we succeeded in obtaining a maximum of 32 merozoites in a corpuscle.

3. Various types of Babesia were seen in these cultures, namely, pyriform, amoeboid, rounded and oval parasites. Division of rounded forms was observed, following the method of gemmation with chromatinic forking. There was evidence, in stained specimens, of direct binary fission.

4. Haemolysis occurred in all the culture tubes.

5. A puppy was successfully inoculated from a 41 hours' culture and succumbed to piroplasmosis on the fifth day.

6. *Babesia canis* is not so easily cultivated by Bass's method as the malarial parasites of man.

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## EXPLANATION OF PLATE XLII

Fig. 1. Microphotograph showing pyriform *Babesia canis* from heart blood of puppy before incubation.

Fig. 2. Shows phagocytosis of four infected red blood corpuscles. From heart blood before incubation.

Fig. 3. Microphotograph showing infected corpuscles containing two and four Babesia. Seven hours' culture.

Fig. 4. Division rosette of eight merozoites from seven hours' culture. Some show loose chromatin.

Fig. 5. Two parasites in process of division by the method of gemmation and chromatin forking. Seven hours' culture.

Fig. 6. Shows mononuclear leucocytosis in piroplasmosis, together with groups of parasites, from a 15 hours' culture.

Fig. 7. Groups of eight and four parasites from a 15 hours' culture.

Fig. 8. Group of sixteen merozoites from a 24 hours' culture.

Fig. 9. Group of sixteen merozoites from a 48 hours' culture, showing increase in size.

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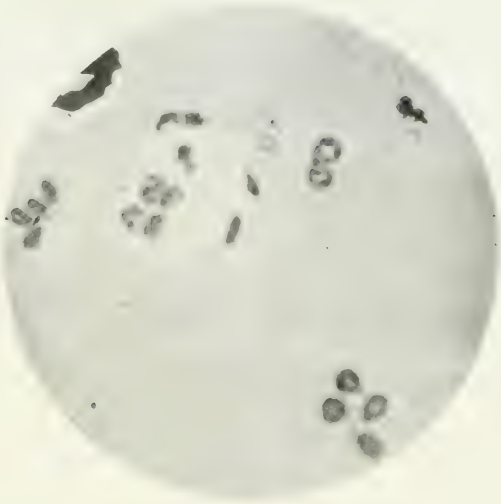

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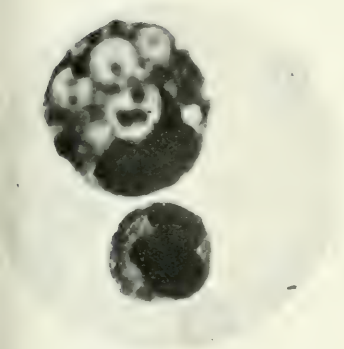

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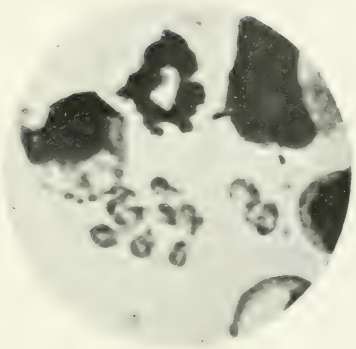

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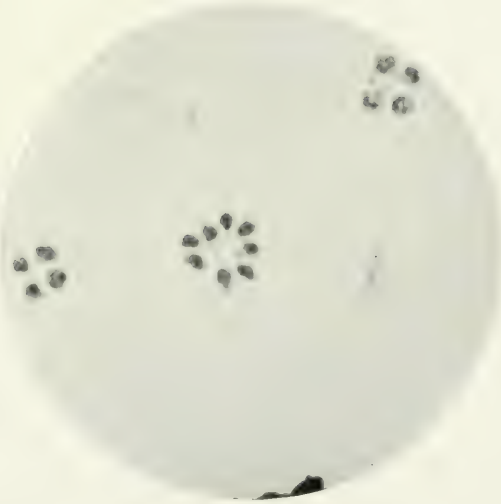

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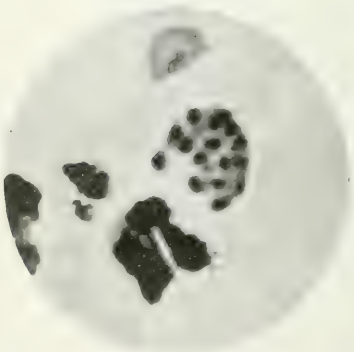

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CULTURAL FORMS OF *BABESIA CANIS*

*J. G. Thomson and H. B. Fantham, photo.* *C. Tinling & Co., Ltd., Imp.*